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A *prfA* Transposon Mutant of *Listeria monocytogenes* F2365, a Serotype 4b Strain, Is Able To Survive in the Gastrointestinal Tract but Does Not Cause Systemic Infection of the Spleens and Livers of Intragastrically Inoculated Mice

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***prfA* is a member of the Crp/Fnr family of global regulatory genes in *Listeria monocytogenes* that has been shown previously to regulate several key virulence determinants both in vitro and in parenterally inoculated laboratory rodents. However, the role of *prfA* in the ability of *L. monocytogenes* to cause infection via the gastrointestinal (GI) tract has not been clearly established. In this study, we used a *prfA* transposon mutant of *L. monocytogenes* F2365, a serotype 4b strain, to assess the role of *prfA* in the pathogenesis of gastrointestinal listeriosis in mice. We found that the *prfA* mutant was able to survive in the GI tract (i.e., cecum) of mice, albeit in numbers somewhat less than those of the wild-type parent strain of *L. monocytogenes*. However, mice inoculated with the *prfA* mutant did not exhibit systemic infection of the spleen and liver, as was noted for mice inoculated with the wild-type parent strain. Survival of the *prfA* mutant in synthetic gastric fluid at pH 2.5 or 5 was somewhat reduced compared to that of the wild-type strain, as was its ability to invade and multiply within differentiated human intestinal epithelial cells (Caco-2 cells). Prior infection with the *prfA* mutant gave mice some protection against a subsequent challenge with virulent *L. monocytogenes*, although much less than that gained by prior gastrointestinal infection with the wild-type parent strain. These findings indicate that the global regulatory gene *prfA* is dispensable for colonization of the GI tract in mice but not for systemic infection.**

Listeria monocytogenes is one of the most important and costly food-borne pathogens. Although the number of cases of listeriosis is far less than for salmonellosis or campylobacteriosis, a large proportion of listeriosis cases cause significant morbidity and mortality (an estimated 2,500 cases and 500 deaths in the United States each year) (21, 28). Populations that are at particular risk of listeriosis include the fetuses of pregnant women and adults who are aged, immunosuppressed, or have other underlying medical conditions (13, 21, 28).

Besides its significance as a food-borne pathogen, *L. monocytogenes* has also been widely used as a model intracellular pathogen. Elegant molecular pathogenesis studies by several laboratories have identified key virulence determinants that allow *L. monocytogenes* to invade both leukocytes and nonphagocytic cells, escape from vacuoles prior to the phagolysosomal fusion within the cell, multiply and move freely within the cytoplasm, and induce the formation of membrane protrusions that are taken up by vacuoles in adjacent cells (11). Coordinated regulation of these virulence determinants is, therefore, crucial for the ability of *L. monocytogenes* to invade cells and cause disease in people and animals. Many of the important virulence genes of *L. monocytogenes* are regulated by a global regulatory gene known as *prfA*, a member of the Crp/Fnr family of global regulatory genes (6, 14, 15). It has been shown that *prfA* mutants are not able to invade and multiply within mammalian cells in vitro and are avirulent when inoculated intravenously or intraperitoneally into mice (6, 14, 15, 30, 33). However, there is little information regarding how *prfA* affects the ability of *L. monocytogenes* to compete and survive in the gastrointestinal (GI) tract, translocate across the intestinal mucosa, and disseminate to other organs where it can cause systemic disease.

Our laboratory has developed a mouse model for gastrointestinal listeriosis in which the genetically susceptible A/J mouse strain develops significant systemic infection following peroral inoculation with *L. monocytogenes* at a challenge dose (i.e., approximately 10^6 CFU) that can occur in contaminated food products (8, 9). In the present study, we used this model to investigate how the *prfA* gene affects the virulence of *L. monocytogenes* in the gastrointestinal tract of mice. Our results indicate that a *prfA* transposon mutant lacked the ability to cause systemic infection of the spleen and liver following intragastric (i.g.) inoculation into mice. However, the mutant strain colonized the gastrointestinal tract (i.e., cecum) in significant numbers and occasionally caused low-level bacteremia in some mice. These findings provide new insights into the role of *prfA* in the pathogenesis of gastrointestinal listeriosis and suggest that an attenuated *prfA* mutant of *L. monocytogenes* can colonize the GI tract without causing progressive systemic infection.

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MATERIALS AND METHODS

Bacterial strain. *L. monocytogenes* strain F2365, a serotype 4b cheese isolate from the 1985 Mexican-style soft cheese outbreak (24) in Los Angles, CA, was obtained from the Centers for Disease Control and Prevention (Atlanta, GA).

Construction of the PrfA mutant strain. A mutation was generated in the *prfA* gene of *L. monocytogenes* strain F2365 as described elsewhere (31). Briefly, the entire *prfA* open reading frame and 500 to 600 bp of flanking sequence was amplified from *L. monocytogenes* strain F2365 by using primers 5′-CATTCAC ACCTCGTCAGTATGC and 5′-CTGACCATGGTGGTGTTACTCG. The amplified products were cloned into plasmid pCR2.1TOPO (Invitrogen Corp., Carlsbad, CA) and used as the recipient plasmids for in vitro mutagenesis using the GPS mutagenesis system (New England Biolabs, Inc., Beverly, MA) according to the manufacturer's instructions. A cloned *prfA* gene bearing a modified Tn*7* (Sp^r) insertion, located 44 base pairs downstream of the *prfA* start, was identified, moved into plasmid pCON1 (2), and recombined back into the strain F2365 chromosome (15). The chromosomal integration was verified by PCR amplification of a product equivalent in size to the mutagenized gene bearing the spectinomycin-resistant transprimer, using forward and reverse nested primers (data not shown) designed from sequences outside of the mutagenized sequence.

Construction and testing of the β-glucuronidase promoter fusion. The promoter region of *plcA* was amplified from *L. monocytogenes* strain F2365 chromosomal DNA using the primers plcAfor (5′-AGGATCCTGGGTTTCACTCT CCTTCTAC) and plcArev (5′-AGTCGACGGCCCCCTCCTTTGATTAG). Amplified products were cloned in front of the *Escherichia coli uidA* gene of plasmid pMLK117 at the BamHI/SalI sites (20). The promoter::*uidA* fusion was transferred to plasmid pHP13 at the EcoRI/HindIII sites and transformed into both *L. monocytogenes* strain F2365 and the *prfA* mutant of this strain. Plasmids were verified in *L. monocytogenes* by colony PCR using the primer plcAfor paired with a primer, gusbeg (5′-CCACCAACGCTGATCAATTCC), designed from the *E. coli uidA* gene. Promoter activities of strain F2365 and the *prfA* mutant strain were compared by assaying for β-glucuronidase activity. Overnight, shaken cultures, grown in 2 ml of brain heart infusion (BHI) broth containing 50 μg/ml spectinomycin (as needed) and 10 μg/ml chloramphenicol, were diluted 1:50 in fresh BHI broth containing 10 μg/ml chloramphenicol and incubated at 37°C. A 2-ml sample was collected for each culture at an optical density at 600 nm of 0.3 and washed in 1 ml sonication buffer (50 mM $NaPO_4$, pH 7.0), and the pellets were frozen. Pellets collected from three independent trials were thawed, resuspended in 0.8 ml sonication buffer, and sonicated in bursts of 20 s on and 10 s off for 6 min using a Sonicator Cell Disruptor (model XL2020; Heat Systems, Farmingdale, NY) on setting 3. Supernatants from each of these trials were assayed in duplicate for β-glucuronidase activity as described and for total protein, using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL) (20). Promoter activities, in nanomoles of *p*-nitrophenol released per minute per milligram of total protein, were calculated from the results of duplicate assays of three individual samples from three independent trials and analyzed using Student's *t* test.

Preparation of *L. monocytogenes* for mouse and Caco-2 cell infection experiments. *L. monocytogenes* was inoculated into BHI broth and incubated for 20 h with shaking at 22°C until mid-log-phase growth was reached. The cells were recovered by centrifugation and resuspended in an equal volume of phosphate-buffered saline (PBS). The optical density of the bacterial suspension was read with a spectrophotometer, and the CFU of *L. monocytogenes* were extrapolated from a standard curve. Appropriate dilutions of the bacterial suspension were made in sterile PBS to achieve the desired bacterial concentration, which was verified by plate counts on tryptic soy agar supplemented with 5% sheep's blood agar (BBL) in each experiment.

Mouse inoculation experiments. The *prfA* mutant, MSF1378, and the wild-type parent strain, F2365, of *L. monocytogenes* were compared for their virulence in a previously described in vivo model of gastrointestinal listeriosis, using the susceptible A/J mouse strain (8). Female inbred A/J mice were obtained (Harlan Sprague-Dawley, Indianapolis, IN) at 5 to 6 weeks of age and housed under microisolator caps at the School of Veterinary Medicine animal care facility at the University of Wisconsin, Madison. Mice were acclimated for 1 week in this facility before being used in an experiment. Mice received food and water ad libitum until 5 h prior to an intragastric inoculation, at which time food was removed from the cage. This was done to ensure that the stomachs of the mice were not engorged with mouse chow, which could prevent delivery of the listerial inoculum into the stomach and potentially lead to aspiration of the inoculum into the lungs. Mice were anesthetized by intraperitoneal injection with sodium pentobarbital (0.75 to 1 mg per 25-g mouse) (Abbott, Abbott Park, IL) as described previously (9). Once sedation occurred, the listerial inoculum was introduced (in a total volume of 0.2 ml) via a 1.5-in. 24-gauge stainless steel feeding needle attached to a 1-ml syringe. At the indicated time points following inoculation, the mice were humanely euthanatized by asphyxiation with CO_2, followed by exsanguination. Blood was collected into a syringe containing sodium citrate (4%) as an anticoagulant. The blood was then serially diluted in sterile saline and plated in duplicate (0.1 ml) on blood agar plates that were incubated for 48 h at 37°C. The numbers of colonies were counted and then used to estimate the numbers of viable *L. monocytogenes* during bacteremia. The abdominal cavity was aseptically opened, and portions of the spleen and liver were removed, weighed in sterile weigh boats, and placed in sterile tissue grinders that contained 1 ml cold sterile saline (0.85%). The tissues were then homogenized, diluted in sterile saline, and plated in duplicate onto blood agar (spleen, liver, blood, and gall bladder) or modified Oxoid agar (cecum) plates. The plates were allowed to dry and then incubated at 37°C for 48 h. The colonies were counted, and the data were expressed as the mean (± standard error of the mean [SEM]) $\log_{10}$ CFU of *L. monocytogenes* per gram of tissue (wet weight). The limit of detection was 1.0 $\log_{10}$ CFU; tissues that did not yield colonies were assigned a value of 0.95 CFU for calculation of the mean ± SEM.

Caco-2 cell attachment and invasion assay. The ability of *L. monocytogenes* to invade and multiply within the human colonic adenocarcinoma cell line Caco-2 (HTB37; American Type Culture Collection, Manassas, VA) was determined as described previously (27). Caco-2 cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Sigma) without antibiotics and supplemented with 25 mM glucose (Mediatech, Herndon, VA), 10% fetal bovine serum (HyClone, Ogden, UT), 1% nonessential amino acids, 2 mM L-glutamine, and 1 mM sodium pyruvate (Sigma). Individual wells of 24-well cell culture plates (Becton Dickinson, Franklin Lakes, NJ) were seeded with Caco-2 cells at a density of 60,000 cells/well, and the cells were allowed to fully differentiate over an 18- to 21-day incubation period. For attachment and invasion assays, bacteria were prepared as described above and resuspended in Caco-2 cell growth medium. At the time of study, the tissue culture medium was aspirated from each well and replaced with 1 ml of medium containing 1×10^7 CFU of *L. monocytogenes*. To assess bacterial attachment and invasion, the monolayers were incubated at 37°C for 1 h. The medium was removed and the monolayers washed five times with warm (37°C) Hanks' balanced salt solution (HBSS). The infected cells were incubated for an additional 2.5 h in HBSS with 20 μg/ml gentamicin (Sigma) to kill any extracellular listeriae. Preliminary experiments verified that this concentration of gentamicin was effective. The cells were then washed five times with warm HBSS and lysed using 1% Triton X-100 (Acros Organics) in PBS. Serial dilutions of the cell lysates were plated in duplicate on blood agar plates and incubated at 37°C for 48 h. The colonies were counted and the data expressed as the mean (± SEM) $\log_{10}$ CFU per well for four independent experiments. To assess intracellular growth, the infected monolayers were incubated for 24 h in DMEM with the supplements indicated above and 20 μg/ml gentamicin. The medium was then removed, the monolayers were washed five times with PBS, the cells were lysed, and the lysates were plated to estimate CFU as indicated above.

Effects of synthetic gastric fluid on survival of *L. monocytogenes*. Synthetic gastric acid was prepared as described previously (7). The *prfA* mutant and wild-type parent strain of *L. monocytogenes* were prepared as described above, washed, and suspended in PBS at a concentration of 10^7 CFU/ml. The listerial suspensions (0.25 ml) were placed into triplicate wells in a 24-well tissue culture plate, and then 2.25 ml of gastric acid fluid was added to each well. The fluid in the wells was mixed by gentle circular rotation of the plate, and then the plate was placed in an incubator at 37°C. At appropriate intervals, samples were removed (0.1 ml) and plated on sheep's blood agar plates. The plates were incubated at 37°C for 24 h and the colonies counted. The results are expressed as the mean (± SEM) $\log_{10}$ CFU of *L. monocytogenes* per ml.

Statistical analysis. Data were analyzed by analysis of variance using GraphPad Prism version 4.0 (GraphPad Software, Inc., San Diego, CA). If a significant *F* value was obtained ($P < 0.05$), the Tukey-Kramer test was performed to determine whether the means of treatment groups differed from those of controls. Statistical significance for all comparisons was set at a *P* value of <0.05.

RESULTS

Intragastric inoculation of a *prfA* mutant of *L. monocytogenes* does not result in systemic infection. Growth of the *prfA* mutant in BHI broth at 22°C was indistinguishable from that of the wild-type parent strain (data not shown). The *prfA* mutant lacked hemolytic activity on both horse blood and sheep's blood agar (data not shown). Also, the *prfA* mutant displayed

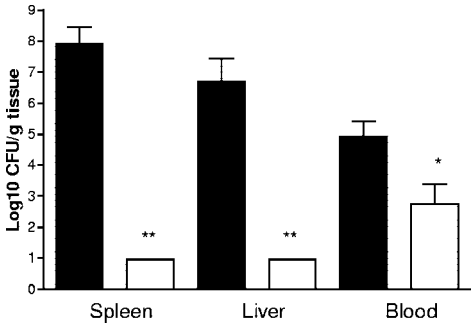

FIG. 1. The *prfA* mutant (□) is avirulent, compared to the wild-type parent strain (■) of *L. monocytogenes*, for i.g.-inoculated mice. Female A/J mice were inoculated i.g. with approximately 10^7 CFU of the indicated strains of *L. monocytogenes* as described in Materials and Methods. Three days later the mice were euthanized, and the numbers of viable CFU in the blood and in homogenates of the spleen and liver were determined by plating on blood agar. The limit of detection was 1.0 $\log_{10}$ CFU. Samples that did not yield colonies were assigned a value of 0.95 $\log_{10}$ CFU for calculation of the mean (± SEM) CFU per gram of tissue or per milliliter of blood for six mice per group. *, $P < 0.05$; **, $P < 0.01$.

less *plcA* promoter activity than F2365 (5.16 ± 0.97 versus 500.09 ± 152.88 nmol/min/μg, respectively), with the activity for the *prfA* mutant being similar to that of the negative control ($P < 0.01$). Both responses are expected because of the known regulation of *hly* and *plcA* by *prfA* (6, 14, 23). We then compared the abilities of the *prfA* mutant and the otherwise isogenic wild-type parent strain to cause systemic infection following i.g. inoculation into A/J mice. Inoculation of mice with approximately 10^7 CFU of the wild-type strain of *L. monocytogenes* caused significant systemic infection, as demonstrated by recovery of viable listeriae from the spleen, liver, and blood (Fig. 1). In contrast, few or no CFU were recovered from the spleens and livers of mice inoculated with the *prfA* mutant (Fig. 1). However, we did isolate viable listeriae from the blood of four of six mice inoculated with the *prfA* mutant. This was surprising, because in our experience we generally recover viable listeriae from the blood only when there are large numbers of CFU of *L. monocytogenes* in the spleens and livers of infected mice (8, 9).

We therefore decided to investigate further the ability of the *prfA* mutant to survive within the gastrointestinal tract, translocate across the intestinal mucosa, and cause systemic disease. Our results confirmed that the *prfA* mutant was substantially impaired in its virulence when inoculated into the gastrointestinal tract. As shown in Fig. 2, i.g. inoculation with the wild-type parent strain of *L. monocytogenes* caused significant systemic infection at challenge doses of as low as 10^4 or 10^5 CFU, and at a challenge dose of 10^6 CFU caused severe systemic infection that would be expected to lead to the demise of the animals if they had not been euthanized on day 3. At the challenge dose of 10^6 CFU we also recovered large numbers of organisms from the blood and gall bladders of mice inoculated with the wild-type parent strain. In contrast, we did not recover viable listeriae from the spleens or livers of mice inoculated i.g. with 10^6 or 10^8 CFU of the *prfA* mutant. We did recover a few CFU from the spleens (three of five mice) and livers (two of

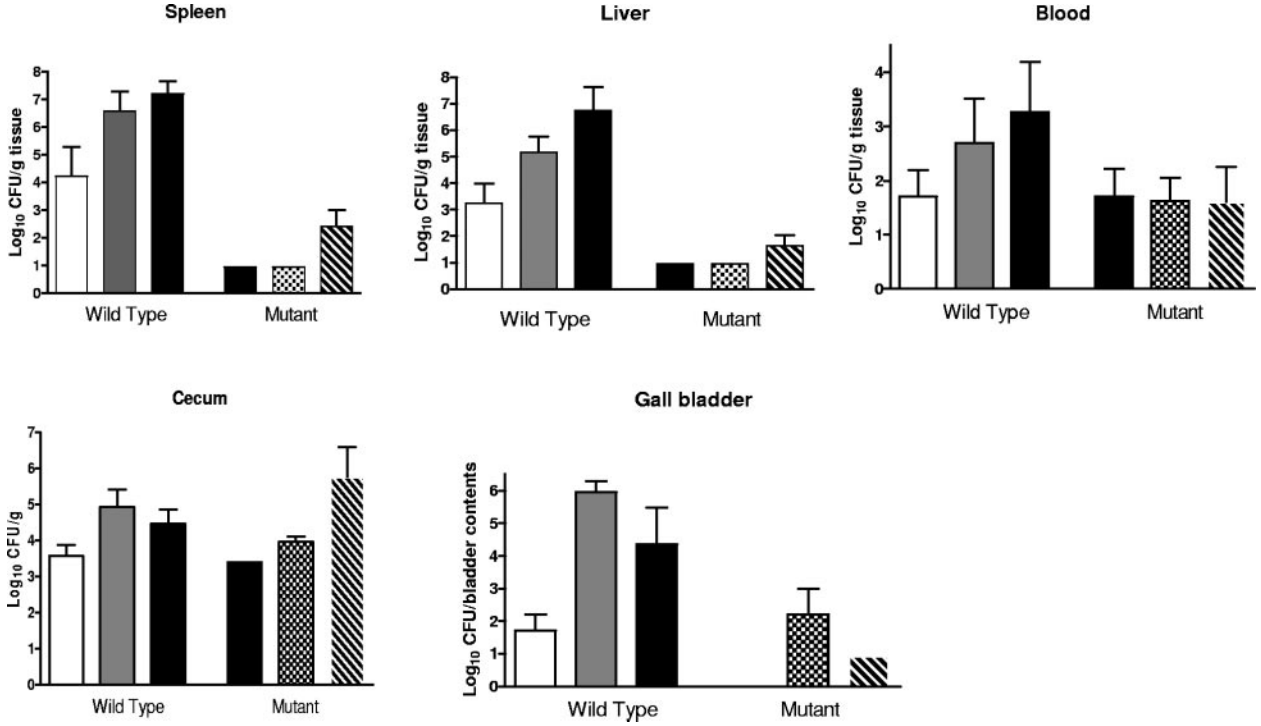

FIG. 2. Comparison of the recovery of viable listeriae from the spleens, livers, blood, ceca, and gall bladders of mice at 3 days after i.g. inoculation with the indicated number of CFU of the wild-type parent (10^4, 10^5, and 10^6 CFU) or *prfA* mutant (10^6, 10^8, and 10^9 CFU) strain of *L. monocytogenes*. Results are the mean (± SEM) $\log_{10}$ CFU per gram of tissue or per milliliter of blood for six mice per group. Data for the gall bladders are per entire organ homogenate.

five mice) of some mice inoculated with a challenge dose of 10^9 CFU of the *prfA* mutant, although these were much fewer than the CFU recovered from mice inoculated with a 1,000-fold-lower challenge dose (10^6 CFU) of the wild-type parent strain. Likewise, the numbers of the *prfA* mutant strain recovered from the blood of some mice (two of six) were also near the limits of detection (10 CFU/ml) and reflected a low-level bacteremia. We recovered a few listeriae from the gall bladders of two of five mice inoculated with 10^8 CFU of the *prfA* mutant but not from those inoculated with the other challenge doses.

Colonization of the gastrointestinal tract following i.g. inoculation of *prfA* mutant or wild-type *L. monocytogenes*. We observed colonization of the gastrointestinal tract by the wild-type parent strain of *L. monocytogenes*, as reflected in the recovery of viable listeriae from the ceca of mice inoculated with a challenge dose of 10^4 or 10^6 CFU (Fig. 2). We also recovered substantial numbers of the *prfA* mutant from the gastrointestinal tract (i.e., cecum), despite its inability to cause systemic infection. The numbers of listeriae recovered from the ceca were greater for mice inoculated with 10^8 or 10^9 CFU of the *prfA* mutant. Thus, intestinal colonization, but not systemic infection, occurs in mice intragastrically inoculated with the *prfA* mutant strain of *L. monocytogenes*.

We compared the abilities of the *prfA* mutant and wild-type strains of *L. monocytogenes* to persist in the GI tract by inoculating mice with 10^6 CFU of either strain and then monitoring fecal shedding and cecal colonization for a 10-day period. We observed similar shedding for the *prfA* mutant and wild-type strains except at day 2, when CFU in the feces were much greater for mice inoculated with the wild-type strain (Fig. 3). This is a time point at which mice inoculated with the wild-type *L. monocytogenes* were experiencing a high listerial burden in the spleen and liver. Whether the greater number of CFU in the feces at day 2 reflects multiplication in the GI tract or possible transport of listeriae in bile from the gall bladder back into the intestinal tract is not clear at this time. From day 4 onwards, fecal shedding generally decreased over time in mice inoculated with either strain, although we still recovered a few CFU at day 10. We recovered greater numbers of CFU of the wild-type strain than of the *prfA* mutant of *L. monocytogenes* from the ceca of mice at 5 days after i.g. inoculation, with the numbers of CFU recovered from mice inoculated with either strain being near the limits of detection at day 10. These data suggest that the *prfA* mutant strain can colonize the GI tract in a manner similar to that for wild-type *L. monocytogenes*, despite its inability to cause systemic infection.

Effect of prior i.g. inoculation with a *prfA* mutant on resistance to i.g. challenge with wild-type *L. monocytogenes*. We next asked whether continued colonization of the gastrointestinal tract by the *prfA* mutant strain might impart enhanced resistance to subsequent challenge with the wild-type parent strain of *L. monocytogenes*. To address this question, mice were inoculated i.g. with 10^8 CFU of the *prfA* mutant. Eighteen days later, these mice and naive (control) mice were inoculated i.g. with the wild-type parent strain of *L. monocytogenes* (challenge dose of 6.7 $\log_{10}$ CFU). Both immunized and nonimmunized mice exhibited mild clinical signs of infection (lethargy and ruffled fur) within 3 days, at which time they were euthanatized. When we compared the numbers of listeriae present in the spleens, livers, blood, and ceca from immunized and non-

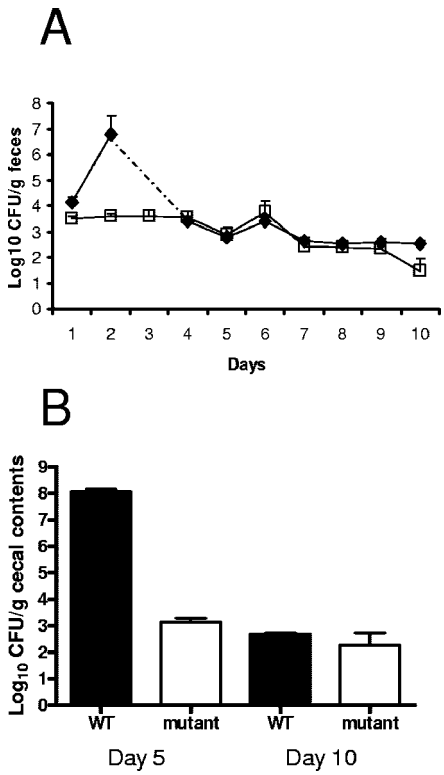

FIG. 3. Fecal shedding (A) and recovery of listeriae from the ceca (B) of mice inoculated i.g. with 10^6 CFU of the wild-type (WT) parent or *prfA* mutant strain of *L. monocytogenes.* Fresh fecal pellets were collected daily into a sterile tube from mice and homogenized in 1.0 ml of PBS. The homogenates were plated on modified Oxoid agar and incubated at 37°C. Ceca were removed, homogenized in sterile PBS, and plated on Oxoid agar as described in the text. Results are the mean (± SEM) $\log_{10}$ CFU per gram of fecal pellet or cecal tissue (wet weight). Results from mice inoculated with the two strains are similar except at day 2 (fecal CFU) and day 5 (cecal CFU), where they are significantly different ($P < 0.05$). Results of fecal shedding for the mice inoculated with the wild-type strains are not illustrated for day 3 because the mice did not defecate when handled, perhaps reflecting the severity of infection on that day.

immunized mice, we found that mice previously infected with the *prfA* mutant harbored fewer listeriae in the spleen and liver than did nonimmunized mice (Fig. 4). Likewise, there were fewer listeriae recovered from the blood, although the numbers of CFU approached the limits of detection. In contrast, comparable numbers of wild-type *L. monocytogenes* were recovered from the ceca of immunized and nonimmunized mice. Thus, prior infection with the *prfA* mutant resulted in limited protection against systemic infection with wild-type *L. monocytogenes*, without preventing the latter from colonizing the GI tract (i.e., cecum). For comparative purposes, we also immunized mice by i.g. inoculation of wild-type. *L. monocytogenes*, followed by challenge with the same parent strain approximately 2 weeks later. In this case, strong protection against systemic infection was elicited, as evidenced by virtually no recovery of viable *L. monocytogenes* from the spleen and liver (Fig. 5). However, once again we recovered similar numbers of listeriae from the ceca of immunized and nonimmunized mice. These findings suggest that prior gastrointestinal listeriosis re-

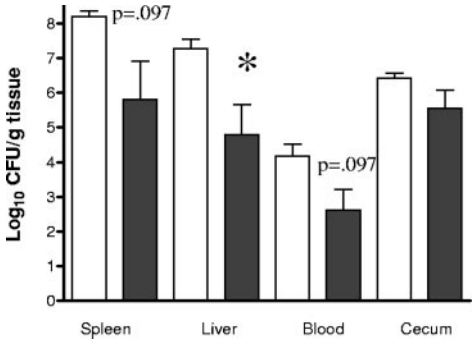

FIG. 4. Prior i.g. infection with the *prfA* mutant strain of *L. monocytogenes* provides limited protection against subsequent i.g. challenge with the wild-type parent strain of *L. monocytogenes*. Mice that had been inoculated previously with 10^8 CFU of the *prfA* mutant strain of *L. monocytogenes* (■) and naive control mice (□) were challenged i.g.18 days later with 5×10^6 CFU of wild-type *L. monocytogenes*. Three days later the mice were euthanized, and the mean (± SEM) $\log_{10}$ CFU per gram of tissue or per milliliter of blood was determined (six mice per group).

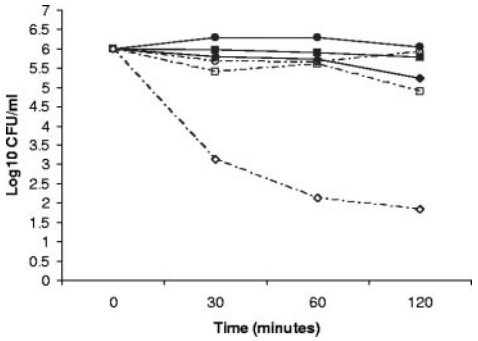

FIG. 6. Survival of the *prfA* mutant (open symbols) and wild-type parent strain (closed symbols) of *L. monocytogenes* in synthetic gastric fluid adjusted to pH 2.5 (◇, ◆), 5 (□, ■), or 7 (○, ●) as described in Materials and Methods. Approximately 2.5×10^6 CFU of the respective strains of *L. monocytogenes* was suspended in 2.5 ml (final volume) of synthetic gastric fluid in a 24-well tissue culture plate. At 30, 60, and 120 min of incubation at 37°C, samples were removed (0.5 ml), diluted, and plated on blood agar. Results are the mean (± SEM) $\log_{10}$ CFU per milliliter (three wells per test condition).

sults in protection against systemic infection, which is dependent on the severity of the initial infection, but does not significantly affect the ability of *L. monocytogenes* to colonize the cecum.

Resistance to synthetic gastric fluid and invasion of Caco-2 cells in vitro. We then compared the *prfA* mutant and wild-type *L. monocytogenes* strains for two in vitro parameters related to gastrointestinal virulence: (i) the ability to survive in synthetic gastric fluid and (ii) the ability to invade human intestinal epithelial cells (i.e., Caco-2 cells). For the first effort, we suspended 7 $\log_{10}$ CFU/ml of log-phase *prfA* mutant or wild-type *L. monocytogenes* in synthetic gastric fluid (7) whose pH was adjusted to 7, 5, or 2.5. As indicated in Fig. 6, we recovered slightly lower numbers of the *prfA* mutant than of the wild-type parent strain from synthetic gastric fluid at pH 5. The difference between the two strains was much greater at pH 2.5, where approximately 3 $\log_{10}$ fewer CFU of the *prfA* mutant strain were recovered after a 30-minute incubation in synthetic gastric fluid. We also found that the *prfA* mutant had less ability to invade fully differentiated Caco-2 cell monolayers following a 1-h incubation in vitro than did wild-type *L. monocytogenes* (Fig. 7, left). The reduced invasive ability of the *prfA* mutant corresponded with its decreased expression of InlA when grown in BHI broth as assessed by Western blotting (data not shown). We recovered fewer CFU from monolayers infected with the *prfA* mutant and incubated for 24 h in medium with gentamicin to prevent extracellular multiplication of listeriae than from monolayers infected with the wild-type parent (Fig. 7, right). However, the CFU recovered from Caco-2 cell monolayers infected with either strain increased during the 24-h incubation period.

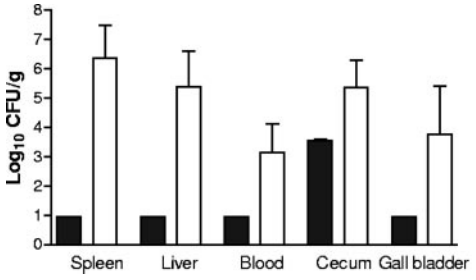

FIG. 5. Prior i.g. infection with the wild-type parent strain of *L. monocytogenes* provides strong protection against subsequent i.g. challenge with the same strain of *L. monocytogenes*. Mice that had been inoculated previously with 10^5 CFU of the wild-type strain of *L. monocytogenes* (■) and naive control mice (□) were challenged i.g. 14 days later with 5×10^6 CFU of wild-type *L. monocytogenes*. Three days later the mice were euthanized, and the mean (± SEM) $\log_{10}$ CFU per gram of tissue (spleen, liver, or cecum), per milliliter of blood, or per entire organ (gall bladder) was determined (six mice per group).

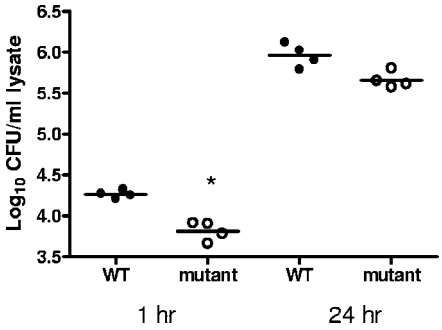

FIG. 7. Invasion (left) and intracellular multiplication (right) of the *prfA* mutant and wild-type (WT) parent strains of *L. monocytogenes* in differentiated Caco-2 cells. Caco-2 cells were incubated for 1 h at 37°C with 1 ml of medium containing 1×10^7 CFU of *L. monocytogenes*. Following incubation, the medium was removed, the monolayers were washed five times with HBSS, and the infected cells were incubated for an additional 2.5 h in HBSS with 20 μg/ml gentamicin (Sigma) to kill any extracellular listeriae. The cells were washed five times with warm HBSS and lysed with 1% Triton X-100 (Acros Organics) in PBS. Serial dilutions of the cell lysates were plated in duplicate on blood agar plates and incubated at 37°C for 48 h. To assess intracellular listerial growth, the infected monolayers were incubated for 24 h in DMEM with the supplements indicated above and 20 μg/ml gentamicin. Results are the mean (± SEM) $\log_{10}$ CFU per milliliter (three wells per test condition) from one representative experiment of three that were performed.

DISCUSSION

We have previously identified the A/J mouse strain as being relatively susceptible to gastrointestinal infection with *L. monocytogenes* (8). Although this model possesses some limitations, it does enable us to investigate environmental conditions or host-pathogen interactions that influence the pathogenesis of listeriosis in the GI tracts of mice.

The results of the present study indicate that the global regulatory gene *prfA* is required for the full expression of virulence by *L. monocytogenes* when inoculated into the murine gastrointestinal tract. *L. monocytogenes* that lacked a functional *prfA* gene had a greatly reduced ability to translocate across the intestinal epithelium and cause systemic disease, although low-level infection of the blood or gall bladder was observed in a few mice. This observation was not unexpected, because *prfA* strongly regulates *hly* (14, 23), which is required for the virulence of *L. monocytogenes* in the gastrointestinal tracts of mice (29). We recover few CFU from the spleens or livers of mice inoculated i.g. with large numbers (10^9 CFU) of the *prfA* mutant (Fig. 2), even during the first 8 h after inoculation (data not shown). These data suggest that either few viable cells of the *prfA* mutant reach the spleen and liver or they are rapidly inactivated after reaching those sites.

The *prfA* mutant did not demonstrate a similar impairment in its ability to survive within the gastrointestinal tract. Mice inoculated with the *prfA* mutant harbored substantial numbers of listeriae in their ceca, albeit less than those in mice inoculated with the wild-type strain, at 5 days after inoculation (Fig. 3B). Fecal shedding by mice inoculated with the *prfA* mutant or wild-type *L. monocytogenes* was similar during the same time period (Fig. 3A). The exception was a spike in CFU of wild-type *L. monocytogenes* in the feces of mice on day 2, when the listerial burden was great in the internal organs (spleen, liver, and gall bladder) of these mice. Whether this represents local multiplication of wild-type *L. monocytogenes* in the gut or reseeding of *L. monocytogenes* in bile or the gall bladder is not clear at this time. These findings suggest that the absence of *prfA*, and thus the expected downregulation of virulence determinants required for systemic infection in vivo (*hly*, *plcA*, *mpl*, *actA*, and *plcB*), is not essential for the ability of *L. monocytogenes* to survive in the gastrointestinal tract.

These data suggest that other factors exert a greater influence on the ability of *L. monocytogenes* to survive and compete within the murine gastrointestinal tract. It has been reported that expression of bile salt hydrolase (*bsh*) activity by *L. monocytogenes* is partially regulated by *prfA* (10), which one would expect to influence the ability of listerial cells to survive within the GI tract. To some extent, our data are consistent with this possibility, since the *prfA* mutant survives less well in synthetic gastric fluid in vitro (especially at pH 2.5) and lower numbers of viable *prfA* mutant cells were recovered from the ceca of mice. However, *bsh* is also regulated by sigma B, which likely explains the partial resistance of the *prfA* mutant to bile salts in the synthetic gastric fluid and its ability to survive in the murine GI tract. These observations suggest that *prfA* regulation of bile salt hydrolase activity might increase the fitness of *L. monocytogenes* in the GI tract without being essential for intestinal colonization in mice. The precise pH of the murine stomach is not known, but it has been estimated to be in the range of pH 2.5 to 4.5 depending on whether the animal has been fasted or fed, with the latter generally stimulating gastric acid secretion (32). Perhaps the residence of the listerial cells in the low-pH environment of the stomach is of short duration in our i.g. inoculation model. Once the listerial cells pass into the duodenum, low pH is not a factor and other bacterial activities are likely more important for survival. It should also be noted that the present study assessed only the numbers of *L. monocytogenes* recovered from a single portion of the gastrointestinal tract (i.e., cecum). It is possible that interactions between *L. monocytogenes* and the host in other sections of the intestinal tract might yield greater differences in numbers of listeriae between the *prfA* mutant and wild-type *L. monocytogenes*.

Some comment should also be made regarding our mouse model. Previous reports from other laboratories identified the importance of the surface protein internalin for the ability of *L. monocytogenes* to bind to E-cadherin on the basolateral surface of human intestinal epithelial cells (Caco-2 cells) (22). Because mouse and human E-cadherins differ at an amino acid that is critical for optimal binding of internalin and transfection of mice with human E-cadherin increases the severity of gastrointestinal listeriosis, there has been a focus on the internalin–E-cadherin receptor-ligand system as the key determinant in the pathogenesis of gastrointestinal listeriosis (11, 22). However, even under optimal conditions for the internalin–E-cadherin receptor-ligand system (i.e., the human E-cadherin transgenic mouse or the guinea pig) (22), the numbers of *L. monocytogenes* needed to cause significant systemic infection following gastrointestinal inoculation far exceeds what would normally be present in a contaminated food product (13, 21, 28). These findings suggest that one or more of the other many surface proteins of *L. monocytogenes* (4, 16, 26) might be involved in its ability to translocate across the intestinal tract and cause systemic disease. Indeed, a recent report identified at least one additional surface protein (auto) that can facilitate gastrointestinal invasion by *L. monocytogenes* (5).

In the present study we did not identify the mechanism responsible for the observed low virulence of the *prfA* mutant. The *prfA* locus is part of the Crp/Fnr family of global regulatory genes that are involved in the bacterial cell response to oxidative stress and other environmental conditions. It is part of the pathogenicity island of *L. monocytogenes* and, as such, regulates a number of important virulence determinants, including *inlA*, *hly*, *actA*, and others (6, 14, 23, 30). Although we did not perform a detailed molecular analysis of these genes in the *prfA* mutant, we did note that it was devoid of hemolytic activity on horse or sheep's blood agar and that expression of *plcA* was greatly reduced, as would be expected. Thus, it is not surprising that the *prfA* mutant was attenuated in virulence, as has been reported previously for mice inoculated parenterally with various *prfA* mutants (15, 18). What is novel and unexpected in the present report is the ability of the *prfA* mutant to persist, in numbers only somewhat less than those of the wild-type parent, in the gastrointestinal tracts of mice. This observation indicates that expression of the full complement of virulence genes regulated by *prfA* in *L. monocytogenes* is not essential for its ability to survive within the gastrointestinal tract. Our recovery of the *prfA* mutant from the gall bladders of mice inoculated only with the highest challenge dose (i.e., 10^9

CFU) is informative. It has been reported that *L. monocytogenes* lacking *hly* can survive extracellularly in the gall bladders of mice (19). However, we did not find this to be the case, with any consistency, for mice inoculated with the *prfA* mutant, which is deficient in expression of *hly*, *plcA*, and presumably other virulence genes of *L. monocytogenes*. Whether the listerial cells are located predominantly in the lumen of the GI tract or within the intestinal mucosa is not clear. However, in previous studies we have not observed obvious lesions or intracellular bacteria in intestinal tissues of mice (unpublished observations)

The *prfA* mutant retained the ability to invade and multiply within Caco-2 cells, albeit less efficiently than wild-type cells, which suggests that it might also be able to invade and survive within intestinal epithelial cells in vivo. Although invasion and survival within Caco-2 cells by a *prfA* mutant might seem surprising, there are several possible explanations. First, *prfA*-regulated virulence gene expression is greater in macrophages than in epithelial cells (3). In keeping with this possibility, one might predict that *prfA* is important in limiting systemic infection in the spleen and liver (as we observed) but less important for infection of epithelial cells lining the gut mucosa. These observations are also consistent with a previous report that Caco-2 cells did not differ substantially in their cytokine transcriptional response to incubation with wild-type or *prfA* mutant *L. monocytogenes* (1). Although invasion of human and murine intestinal epithelial cells differs in its reliance on internalin (11, 22), InlA is regulated by both *prfA*-dependent and -independent pathways (11, 12, 17, 18, 25, 30). Perhaps the latter was of greater importance for invasion of and intracellular multiplication in Caco-2 cells by the *prfA* mutant in the present study.

Clearance of a gastrointestinal infection with the *prf*A mutant reduced somewhat the severity of systemic infection in mice that were subsequently challenged with wild-type *L. monocytogenes.* Whether the low level of protection that we observed was due to a local immune response in the GI tract or to initiation of a systemic immune response in the spleen was not examined in the present study. Certainly the degree of protection that was engendered by prior infection with the *prfA* mutant paled in comparison with that resulting from prior clearance of a gastrointestinal infection with wild-type *L monocytogenes*. Prior infection with either the *prfA* mutant or wild-type *L. monocytogenes* did not prevent colonization of the gastrointestinal tract following a second i.g. challenge with wild-type *L. monocytogenes.* These findings suggest that efforts to use immunization to prevent carriage of *L. monocytogenes* in the GI tract could be problematic.

In summary, this study provides evidence that the global regulatory gene *prfA* is not essential for colonization of the gastrointestinal tract in mice. However, in its absence, *L. monocytogenes* is largely restricted to the gastrointestinal tract and fails to cause systemic infection of the spleen and liver, the target organs that normally harbor the greatest numbers of listeriae following experimental challenge. These findings suggest that distinct regulatory events are primarily important for gastrointestinal and systemic infection of mice with *L. monocytogenes*, with the former being *prfA* independent and the latter *prfA* dependent.

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